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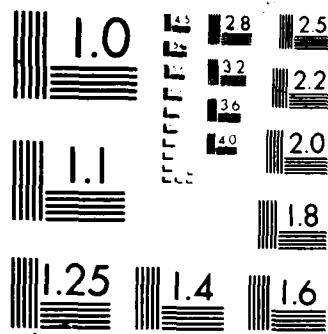UNCLASSIFIED DAMD17-85-C-5020 F/G 6/1 NL

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STUDIES ON THE PATHOGENESIS OF HEPATITIS A AND
FEASIBILITY STUDIES ON A HEPATITIS A VACCINE

ANNUAL REPORT

Elvera Ehrenfeld, Oliver C. Richards and
Donald F. Summers

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19. ABSTRACT (Continue on reverse if necessary and identify by block number) The objectives of this work are to further our knowledge of the pathogenesis of hepatitis A virus (HAV) infection in man, and to develop recombinant expression vectors for hepatitis A virus antigens that can be used to stimulate mucosal immunity. Two viral cDNA sequences encoding different forms of capsid proteins have been successfully cloned into several bacterial expression vector plasmids, transformed into E. coli and analyzed. Expression tests are still in progress. An in situ hybridization procedure has been developed, using probes developed in our cloning experiments. This procedure can readily detect HAV-infected cultured cells, and is now ready to be applied to fixed animal tissue sections. HAV is being grown in small scale in our laboratory, and a "dot blot" hybridization procedure has been developed to understand the course of infection. A variety of human acute and convalescent sera have been obtained for analysis of the anti-HAV antibody specificity by immunoblot tests against virus, viral capsid proteins and infected cell extracts. These experiments should help advance our understanding of the pathogenesis of enteric virus infections and begin to elucidate new strategies for the construction of viral vaccines.

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Summary

The objectives of this work are to further our knowledge of the pathogenesis of hepatitis A virus (HAV) infection in man, and to develop recombinant expression vectors for hepatitis A virus antigens that can be used to stimulate mucosal immunity. Two viral cDNA sequences encoding different forms of capsid proteins have been successfully cloned into several bacterial expression vector plasmids, transformed into E. coli and analyzed. Expression tests are still in progress. An in situ hybridization procedure has been developed, using probes developed in our cloning experiments. This procedure can readily detect HAV-infected cultured cells, and is now ready to be applied to fixed animal tissue sections. HAV is being grown in small scale in our laboratory, and a "dot blot" hybridization procedure has been developed to understand the course of infection. A variety of human acute and convalescent sera have been obtained for analysis of the anti-HAV antibody specificity by immunoblot tests against virus, viral capsid proteins and infected cell extracts. These experiments should help advance our understanding of the pathogenesis of enteric virus infections and begin to elucidate new strategies for the construction of viral vaccines.

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A. Statement of the problem.

Hepatitis A virus (HAV) infection, one of the most prevalent infections of man, causes significant morbidity and remains a worldwide public health problem. In this work, we propose to further our knowledge of the pathogenesis of hepatitis A virus infection of man, and to develop recombinant expression vectors for HAV antigens that can be used to stimulate mucosal immunity.

B. Background.

Hepatitis A virus (HAV) is the most common cause of viral hepatitis, accounting for approximately 30,000 reported cases each year during the 1980s Center for Disease Control, 1985). It is a particular problem in certain population groups such as day-care center attendees and employees, shellfish eaters, food handlers, patients and staff in institutions for the mentally retarded, travelers, and homosexuals. Although the mortality is very low, HAV infection can cause considerable morbidity and expense to the individual and family (Storch et a., 1979). Transmission occurs predominantly via the fecal-oral route by both common vehicle and person-to-person modes and peaks prior to onset of clinical illness in the source case making prevention of infection a particularly important public health objective.

Research on the biology of HAV and the pathogenesis of the infection it produces became possible only recently as a result of the successful infection of marmosets (Holmes et al., 1969), visualization of viral particles in stool specimens (Feinstone et al., 1973), and the successful propagation of HAV in tissue culture cells (Provost and Hilleman, 1979). The virus can now be propagated in vitro in a variety of primary, passaged, and transformed cell cultures of human and nonhuman primate origin. Although the infection in cell cultures is not lytic and, therefore, traditional plaque assays to quantify virus are not possible. Viral antigen can be detected in cultured cells by a variety of immunologic methods such as radioimmunoassay, enzyme linked immunosorbent assay, and immunofluorescence and an acccurate determination of virus titers can be made using a newly described radioimmunofocus assay (Lemon et al., 1983). Present characterization of HAV reveals it to be a member of the enterovirus genus of the picornavirus family (Coulepis et al., 1982; Melnick, 1982). It is a 27-nm spherical nonenveloped virus with icosahedral symmetry and bands at 1.34 gm/cm^3 in cesium chloride (Siegl and Frosner, 1978). The genome is a single-stranded plus-sense monocistronic RNA of 2.8×10^6 daltons with a 3' poly(A) tract, encodes at least 11 virus-specified polypeptides VP1, VP2, VP3, and VP4, and is extremely stable to chemical and

physical agents (Locarnini et ai., 1981). Studies of the antigenic relatedness of different strains of HAV have identified only one serotype (Lemon and Binn, 1983). The entire HAV genome has been cloned as cDNA into pBR322 and sequenced revealing little homology with other picornaviruses (von der Helm et al., 1981; Ticehurst et al., 1983; Linemeyer et al., 1985; Baroudy et al., 1985; Najarian et al., 1985).

The information derived from cloning and sequence data can be used to produce synthetic peptides, to express structural proteins in prokaryotic and eukaryotic vectors, and to engineer recombinant bacteria in an attempt to develop an effective vaccine against HAV. As there is no treatment for hepatitis A disease, control and prevention of HAV infection is likely to result only from development and use of such a vaccine. The impact that vaccines can have on common infectious diseases of man has been demonstrated repeatedly by experience with other agents including polio, mumps, measles, and rubella (Hilleman, 1985). The success of a hepatitis A vaccine will depend, in part, on understanding the cellular route of infection and the host's immune response to allow selection and presentation of viral epitopes in an appropriate manner.

Following the development of immunologic assays in the early 1970s to detect virus antigen and antibody, studies of the pathobiology and immune response generated by HAV infection in primates became possible. It was observed that as early as 1 week following oral inoculation of marmosets HAV can be detected in the liver (Provost et al., 1978); in fact, to date replication of virus has been demonstrated only in the liver. Hepatocellular necrosis in HAV infection of chimpanzees and man appears to be limited to the peripheral portal area of the liver lobule (Popper et al., 1980) suggesting that virus is transported to the liver from the intestine via the portal veins and that viremia occurs only after replication of virus in the live-. HAV can also be identified by immunoflourescent staining in bile and finally, only after appearance in the liver and concomitant with the onset of prodromal symptoms, fecal excretion of HAV antigen occurs (Mathiesen et al., 1978). Whether HAV infection of hepatocytes is in itself cytolytic and responsible for disease or whether liver cell necrosis results from the host's immunologic response is not clear. With the onset of aminotransferase elevation and jaundice, excretion of HAV in stool has usually ceased and infected hosts are no longer infectious.

Although HAV has been detected by immunoflourescence in the spleen, abdominal lymph nodes, and renal glomeruli in experimental animals infected percutaneously (Mathiesen et al., 1978), HAV antigen has not been detected in these organs of orally inoculated animals (Mathiesen et al., 1980; Krawczynski et al., 1981). The identification of HAV

antigen in the former instance was felt to be the result of antigen trapping in the form of immune complexes. Despite exhaustive attempts to demonstrate HAV in intestinal epithelial cells of percutaneously and orally inoculated animals by immunocytochemical methods, the presence of HAV antigen in the intestinal mucosa has not been demonstrated. Similar studies using nucleic acid probes have not been reported.

Antibody of the IgM class to HAV appears very early during acute illness and, in fact, may precede the onset of clinical disease and coexist with viremia and infectivity suggesting that such early antibody is nonneutralizing (Bradley, et al., 1977). Although IgM anti-HAV appears transiently and becomes undetectable after 3 to 6 months, IgG anti-HAV develops gradually, persists in high titers for years following HAV infection, and correlates with immunity against reinfection. Study of the specificity of this protective antibody and characterization of neutralization epitopes of viral proteins have been hampered by the slow growing, low-yielding, nonlytic nature of HAV in tissue culture. Structural proteins of HAV have been described using surface-labeling techniques and, as with other picornaviruses, VP1 has been demonstrated to be the most exposed surface component of the virion; VP3 and VP2 are less exposed and VP4 is undetectable (Gerlich and Frosner, .983).

Recent studies with neutralizing monoclonal antibodies have suggested that VP1 contains at least one neutralization site for HAV (Hughes et al., 1984). Immunization of rats with VP1 purified from cell cultures results in the development of a strong antibody response to the isolated VP1 polypeptide as determined by Western blot analysis and immune precipitation, a good antibody response to the whole virus as demonstrated by competitive radioimmunoassay (RIA) and precipitation of HAV, and low titer neutralizing antibody to HAV as demonstrated by cell culture assay (Hughes and Stanton, 1985). These data indicate that denatured VP1 subunit does present at least some neutralizing epitopes to the immune system that are similar to those presented by the intact virus. VP3 can induce antiprotein and neutralizing responses but the antiVP3 serum is not reactive in competitive RIA suggesting that there are epitopes on denatured VP3 which are not normally exposed on the intact virion. Immunization with denatured whole HAV seems to elicit the strongest antiprotein, antiviral, and neutralizing responses (Hughes and Stanton, 1985).

Although recent data indicates that the capsid protein, VP1, and perhaps VP3, is the important target for HAV neutralizing antibodies, there are little data concerning the relative importance of systemic versus local immunity in affording protection. The presence of serum anti-HAV correlates with resistance to reinfection but may be an

epiphenomenon as secretory IgA can be detected in stools of patients with acute hepatitis A and, therefore, may play a role in limiting the duration of infectivity and providing local intestinal immunity to prevent reinfection in cases of reexposure (Yoshizawa et al., 1980; Locarini et al., 1980). Resistance to many infections acquired at the mucosal surface is better correlated with the local synthesis, transport, and secretion of specific secretory IgA antibodies than with systemic antibody (Allardyce and Bienenstock, 1984). One way to test this hypothesis would be through the use of Salmonella typhimurium gal E mutants which are rough-type strains that contain a mutation in the UDP-galactose-4-epimerase gene. Their avirulence is attributable to bacterial lysis that follows the uptake of galactose and its accumulation in the form of galactose-1-phosphate and UDP-galactose; their protective capacity as a live vaccine is attributable to synthesis of immunogenically important cell wall lipopolysaccharides of the smooth-type in the face of exogenous galactose (Germanier ad Furer, 1971). A stable gal E mutant of S. typhi Ty21a, was isolated by Germanier and Furer (1975) and found to protect mice when given intraperitoneally against lethal challenge with S. typhi.

In volunteer studies the S. typhi Ty21a strain cultivated in the presence of 01% galactose was stable, well tolerated, protective against subsequent challenge with virulent S. typhi, and was excreted in stools for only the first few days after oral administration (Gilman et al., 1977). Although no significant systemic antibody response to O, H, or Vi antigens resulted from vaccine administration in these studies, recent data suggest that induction of local immunity may play a role in the protective effect of Ty21a as all immunized subjects produce specific enteric IgA to both lipopolysaccharide and flagellar S. typhi antigens (Cancellieri and Fara, 1985). The Ty21a typhoid vaccine strain has been tested in extensive field trials in Egypt (Wahdan et al., 1982) and Chile (Levine, 1985) and has been found to be highly effective in preventing typhoid fever with a protection rate of 95% lasting at least 3 years. The oral vaccine produced none of the adverse reactions such as fever, local inflammation, and headache, noted frequently with parenteral typhoid vaccine.

The gal E mutant S. typhi strain has been considered as a useful orally administered carrier for antigenic determinants other than those of S. typhi which has the potential to protect against a variety of intestinal infections; one system utilizing this approach has been described (Formal et al., 1981). Immunity to Shigella sonnei, the most common cause of bacillary dysentery, is associated with a type-specific somatic antigen encoded by a large plasmid. With the intention of constructing a bivalent vaccine strain that would be protective against shigellosis due to S. sonnei and typhoid fever, Formal et

al. (1981) conjugally transferred the plasmid-borne cell surface antigenic determinants of S. sonnei to the S. typhi Ty21a vaccine strain. The transconjugant strain was shown serologically to produce the S. sonnei surface antigen as well as S. typhi somatic antigens and, as the parental strain, to remain viable in mouse spleens for less than 3 days following intraperitoneal injections. Furthermore, the nonlethal derivative strain protected mice against lethal challenge doses of either S. typhi or S. sonnei. Thus, the gal E mutant of S. typhi vaccine strain can be successfully modified to produce immunity to at least one other gastrointestinal pathogen in a murine model. As a safe vector to carry genes encoding antigenic determinants of other pathogens and express these genes within the intestinal mucosa, the gal E mutant S. typhi Ty21a strain is attractive for the development of recombinant vaccines against enteric agents for which the local intestinal immune response is felt to be important.

C. Project aims.

1.   1a. To construct recombinant bacterial plasmids that will express hepatitis A virus capsid proteins.

   1b. To transform the gal E mutant Salmonella typhi Ty21a oral vaccine strain with hepatitis A virus recombinant plasmids and evaluate the modified strain's ability to induce a systemic and mucosal immune response.

2.   To analyze extrahepatic tissue from infected animals for hepatitis A virus replication.

3.   To determine the specificity of the human immune response to hepatitis A virus infection.

D. Results.

1.   Construction of recombinant plasmids capable of expressing hepatitis A virus capsid proteins in bacteria.

   We have chosen two HAV capsid-encoding regions for our initial attempts at expression in bacterial vectors. One is a sequence encoding VP1, the polypeptide which we believe represents the major external surface protein and

therefore is likely to contain immunologically important
epitopes.   The other is the sequence encoding about the
entire capsid protein coding region, in the form of a
single, uncleaved polypeptide chain.   We have constructed
and isolated plasmids containing the sequences of
interest, as described below.

a)   pIN-III expression vectors

This vector contains the E. coli lipoprotein gene
promoter, ribosome binding site and translational initiation
codon, plus the sequences coding for the lipoprotein signal
peptide and eight amino acids, followed by a cloning site.
Thus, cloned material should be secreted across the
cytoplasmic membrane as a hybrid protein.   All three reading
frames are available.   In addition, a lac UV5 promoter-
operator has been inserted upstream of the ribosome binding
site so that transcription should require a lac inducer.   In
fact, transcription does occur even in the absence of lac
inducers.

(1)   Total capsid coding sequences.

Two clones of E. coli containing capsid-coding
sequences in pBR322 vectors were obtained from John
Ticehurst at NIH.   These are diagrammed below, with
relevant restriction sites indicated.
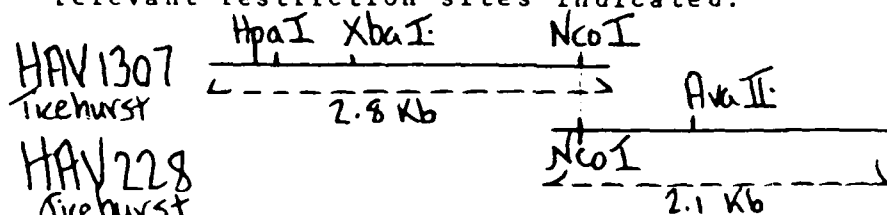
We prepared both of these DNAs, cut each with NcoI and
EcoRI (sequences in pBR322 portion of both plasmids)
religated and isolated a clone containing the HAV
sequences from both plasmids on a single continuous 4.5 kb
insert in pBR322.   This plasmid is called
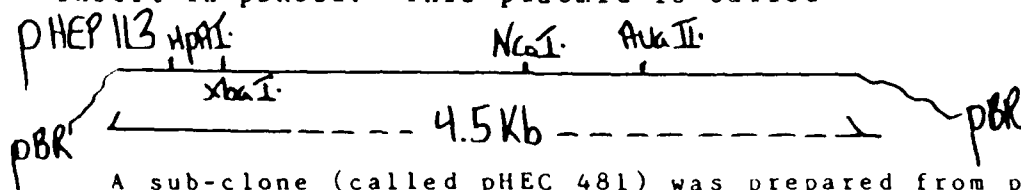
A sub-clone (called pHEC 481) was prepared from pHEP113
that extended from the HpaI site to the AvaII site (3.1
kb) by cutting pHEP113 with these two enzymes, isolating
the fragment from an agarose gel, making blunt ends by
fill-in with Klenow fragment of DNA polymerase, adding
phosphorylated EcoRI linkers, and ligating into the EcoRI
site of pBR322.   Since translational termination codons
remain in the cloned material between the HpaI site and
the putative open reading frame of the hepatitis A virus
capsid proteins, pHEC481 was restricted with XbaI (which
removed about 10 nucleotides from the putative open
reading frame), and blunt-ended, ligated to EcoRI linkers

and re-cloned into pIN-III expression vectors (gift of M. Inouye).

Ligation mixtures were transformed into competent E. coli, and ampicillin-resistant colonies were screened for the presence of chimeric pIN plasmid DNA containing HAV sequences. Forty-nine HAV-positive colonies were isolated and 35 were tested for orientation and expression of HAV proteins. The results from pIN-III C3, calculated to provide the proper reading frame, are shown below.

    25 - wrong orientation
     4 - insert lost
     5 - DNA rearrangement occurred
     1 - correct orientation, but additional pBR322
         sequences were inserted upstream of the HAV
         coding region.
    None expressed HAV protein.

(2) VP1-coding sequences.

The idea of an expression vector that would secrete the expressed HAV sequences is inherently attractive for the purposes of this proposal; thus experiments with the pIN-III expression system were conducted simultaneously for both of the HAV sequences.

Plasmid pHEP 113 (described above) was restricted with HindIII and AvaII, releasing a 1.3 kb fragment of HAV cDNA, which includes the sequence of capsid protein VP1. The fragment was purified from preparative agarose gels, ends modified to terminate with EcoRI linkers by methods similar to those described above, and the DNA was ligated into Eco RI-restricted pBR322. Transformed E. coli yielded a clone, pHAV-518, which contained a plasmid carrying two tandemly repeated VP1 sequences. This insert was isolated and ligated into the pIN-III C2 (calculated to provide the correct reading frame) expression vector.

    Twenty-five pIN/VP1 recombinants were analyzed:
        14 - wrong orientation, single copy of VP1 insert
         5 - wrong orientation, double copy of VP1 insert
         2 - both orientations, multiple copies
         1 - correct orientation, double copy
         3 - lost insert
    None expressed HAV protein.

We have terminated efforts to express HAV sequences in pIN vectors. At this time, it seems possible that continuous expression of these HAV sequences from this vector is lethal, since in both sets of experiments, no clones were isolated that contained the sequences in the right orientation for expression, without some additional DNA rearrangements. However, the constructions we have made in pBR322 and in pIN are useful sources of DNA for rapid movement into other expression vectors.

    pATH .e

This vector (gift of Dr. C. Dieckmann) is designed to express a fusion protein containing the amino terminus of the E. coli tryp E protein, expressed from a strong tryp promoter. It is inducible, but difficult to shut off completely. We have successfully expressed poliovirus proteins from this vector in our laboratory, and thus feel encouraged with the system. We are currently modifying the vector to eliminate the majority of tryp E coding sequences and to construct the right reading frame to accept HAV VP1 sequences. No efforts at expression have yet been made.

(c) pAS

This vector (received from Dr. M. Rosenberg, Smith, Kline and French) expresses from the lambda right promoter, and has the advantage of being completely and totally repressed until manipulated by temperature or drug treatment. We have tested expression of the influenza NS protein from this promoter at 37°C, and find it to be about 50% of that at the maximal induction temperature of 43°C. Thus, it may be adaptable to the human in vivo situation. Cloning of HAV capsid sequences into this vector are now in progress.

1. Expression of hepatitis A VP1 from baculavirus vectors in insect cells.

Although not in our original proposal, we have undertaken a side project to attempt to prepare large amounts of HAV VP1 by expression from a baculavirus vector in insert cells. This expression system (obtained from Dr. Max Summers) has been reported to yield mg amounts of recombinant proteins from a variety of sources, in soluble, active form. We plan to use the VP1 so obtained to induce antibodies in rabbits to provide a needed reagent for our expression studies, and, in addition, we plan to test this VP1 as an immunogen in collaboration with Walter Reed Army Institute of Research (WRAIR).

To date, the insect cell cultures have been established in the laboratory, baculavirus has been grown, and appropriate plasmids containing the baculavirus polyhedron promoter and cloning sites have been prepared. A recombinant plasmid containing the HAV VP1 insert from pHAV-518 has been isolated and characterized by restriction mapping. Transformation experiments with plasmid and viral DNA are in progress.

2. Analysis of tissue for hepatitis A virus genome.

Since the laboratory was not experienced with in situ hybridization techniques, we developed procedures using control and HAV-infected BSC-1 cells, provided by Binn and Machowicki at WRAIR (ref A371 p. 3). The cells had been grown in Lab Tek trays and fixed with PLP at WRAIR. (Our procedures were adapted from Haase et al., Methods in Virology, Vol. VII, pp. 189-226, 1984, and from Stein and

Yanishevsky, Methods in Enzymology, pp. 279-292, 1979).
Slides were treated prior to hybridization with 0.2 N HCl,
2 x SSC at 70°C: and pronase digestion. The probe was
nick-translated, product labeled with ^{35}S-dATP, from a 2.8
kb gel-purified DNA representing HAV capsid region. After
hybridization and washing, slides were coated with Kodak
NTB-2 nuclear track emulsion and stored in the dark.
After 5 days, the slides were developed and fixed, and
cells were stained with giemsa for viewing by light
microscopy. The results showed rather spectacular
cytoplasmic hybridization in the infected cells (4+), over
a slight background in the nuclei and in infected cells.

We have requested infected and uninfected monkey liver
biopsy material from WRAIR in order to try our first
tissue specimen preparation and hybridization in vitro,
but have not yet received any material.

3.   Analysis of human immune response to hepatitis A virus
      infection.

Preliminary experiments were proposed to test human
sera from HAV patients in an immunoblot procedure to
determine which capsid proteins (or non-capsid proteins)
elicit antibodies during natural infection. These
experiments first required a source of virus and infected
cells.

(a) HAV culture and assay.
        BSC-1 cells and HAV (strain HM175) were obtained
from Dr. Binn at WRAIR and brought to our laboratory.
Cells are being propagated regularly, and small-scale
infections have been conducted to expand our virus
pool. A dot hybridization procedure has been developed
to initiate virus replication and to quantitate the
amount of vira RNA in infected cells. Biotinylated
cDNA was first tested as a probe, with avidin linked to
alkaline phosphatase in a colorimetric assay. However,
in our hands, nick-translated, ^{32}P cDNA probe is
significantly more sensitive. Cytoplasmic extracts
from HAV-infected cells were prepared, and dilutions
were spotted onto nitrocellulose, which was
subsequently hybridized with probes prepared from nick-
translated pHAV-113. Control analyses were preformed
with polio cDNA probes prepared together with the HAV
probes, and polio-infected HeLa cell cytoplasm. The
results suggest that there is approximately one half
the amount of viral RNA in 28-day HAV-infected cells as
there is in 5-hour polio-infected cells, amounting to
approximately 5 x 10^{12} virus particles per ml. About
80% of the viral RNA was found in the infected cell
cytoplasm, and only 20% was released into the culture
medium. No cross-hybridization was observed between
HAV and polio RNA with the probes used.

At this time, we are able to produce small-scale and

purify small amounts of virus. We have requested some purified virus for immunoblot analyses (see below) from CDC, but have not yet received an answer. We may have to scale up our own preparations.

(b) Anti-HAV sera.

Samples of convalescent human serum and rabbit anti-sera prepared against intact HAV were obtained from WRAIR. In addition, during a visit with diagnostic virologists from a Madrid, Spain hospital, we obtained some samples of high titer, acute and convalescent human sera.

(c) Immunoblotting of HAV-infected BSC-1 cell cytoplasmic proteins.

We have performed a few Western blots with the WRAIR human antiserum and the rabbit anti-HAV serum, against BSC-1 cell cytoplasmic proteins separated by SDS-PAGE and transferred to nitrocellulose filters. No reaction was observed with the human antiserum; the rabbit serum appeared to react with one polypeptide not present in mock-infected cells. We are in need of purified virus for control immunoblots (see above), and are still in the beginning stages of these investigations.

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